

Molecular Phylogeny, Classification, Evolution, and Detection of Pestiviruses

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Abstract

The genus *Pestivirus* comprises four recognised species: *Bovine viral diarrhoea virus 1* (BVDV-1), *Bovine viral diarrhoea virus 2* (BVDV-2), *Border disease virus* (BDV), and *Classical swine fever virus* (CSFV); and a tentative species, *Pestivirus* of giraffe. Recently, additional pestiviruses have been identified and presumed as novel subgroups/species based on single gene analyses that reveal different relationships among pestiviruses, depending on the genetic regions being analysed and the methods used.

This thesis describes the application of a new strategy—analyzing a combined sequence dataset, and use of different methodologies in inferring pestivirus phylogeny, as a basis for both genetic classification and evolutionary studies. The Bayesian approach is demonstrated to be useful for inferring BVDV-1 phylogeny, where best result was obtained from the analysis of a combined sequence dataset. This strategy and method produce a reliable, well-supported phylogeny of BVDV-1. The whole-genome phylogeny of pestiviruses showed a closer phylogenetic relationship between the atypical pestiviruses and the recognised species BVDV-1 and BVDV-2. In order to obtain a unified classification scheme of pestiviruses, Maximum likelihood and Bayesian analyses of a molecular dataset of 56 pestiviruses and 2089 characters were performed, resulting in an identical, reliable tree topology. Dating of the most recent common ancestor was estimated for major pestivirus lineages and their evolutionary histories were revealed. Based on these results, a new proposal is suggested for the genetic classification of pestiviruses into nine species: BVDV-1, BVDV-2, BVDV-3, *Pestivirus* of giraffe, CSFV, BDV, Tunisian sheep virus, Antelope virus and Bungowannah virus.

As powerful tools of molecular diagnostic virology, two real-time PCR assays have been developed for detection of atypical pestiviruses and CSFV by using TaqMan and primer-probe energy transfer (PriProET) technologies, respectively. Both assays are highly sensitive, specific, reproducible, and useful for the improved detection of two pestiviruses.

Keywords: Bayesian, classification, evolution, Maximum likelihood, pestivirus, real-time RT-PCR

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Contents

List of Publications	7
Abbreviations	8
1 Introduction	9
1.1 Pestiviruses	9
1.2 Viral infection, diseases and their importance	9
1.3 Genome organisation	11
1.4 Phylogeny, classification, and evolution	12
1.5 Molecular detection of pestiviruses	14
2 Aims of the study	17
3 Methodological considerations	19
3.1 Strategy	19
3.2 Neighbour-joining, Maximum likelihood, and Bayesian approach	20
3.3 Dating of the most recent common ancestors	20
3.4 Real-time RT-PCR	21
4 Results and discussion	23
4.1 New strategy and method for inferring BVDV-1 phylogeny	23
4.2 Whole-genome phylogeny of pestiviruses	24
4.3 Pestivirus phylogeny, classification and evolution	25
4.4 Molecular detection of pestiviruses by real-time RT-PCR	28
5 Conclusions	31
References	33
Acknowledgements	39

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Xia, H., Liu, L., Wahlberg, N., Baule, C., Belák, S. (2007). Molecular phylogenetic analysis of bovine viral diarrhoea virus: a Bayesian approach. *Virus Research* 130, 53-62.
- II Liu, L., Kampa, J., Belák, S., Baule, C. (2009). Virus recovery and full-length sequence analysis of atypical bovine pestivirus Th/04_KhonKaen. *Veterinary Microbiology* (under revision).
- III Liu, L., Xia, H., Wahlberg, N., Belák, S., Baule, C. (2009). Phylogeny, classification and evolutionary insights into pestiviruses. *Virology* 385, 351-357.
- IV Liu, L., Xia, H., Belák, S., Baule, C. (2008). A TaqMan real-time RT-PCR assay for selective detection of atypical bovine pestiviruses in clinical samples and biological products. *Journal of Virological Methods* 154, 82-85.
- V Liu, L., Xia, H., Belák, S., Widén, F. (2009). A novel real-time PCR assay based on primer-probe energy transfer for the improved detection of classical swine fever virus (Submitted to *Journal of Virological Methods*).

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Abbreviations

BDV	<i>Border disease virus</i>
BEAST	Bayesian Evolutionary Analysis Sampling Trees
BVDV-1	<i>Bovine viral diarrhoea virus 1</i>
BVDV-2	<i>Bovine viral diarrhoea virus 2</i>
BVDV-3	Bovine viral diarrhoea virus 3
cp	cytopathogenic
CPE	cytopathic effect
cRNA	complementary RNA
CSFV	<i>Classical swine fever virus</i>
Ct	threshold cycle
CV	co-efficiency of variation
DIVA	Differentiation of Infected from Vaccinated Animals
EMEM	Eagle's minimum essential medium
GTR	General Time Reversible
HPD	highest posterior density
ILD	incongruence-length-difference
MCMC	Markov chain Monte Carlo
ML	Maximum likelihood
MRCA	most recent common ancestor
ncp	non-cytopathogenic
NJ	neighbour-joining
ORF	open reading frame
PriProET	primer-probe energy transfer
RT	reverse transcriptase
S.D.	standard deviation
T _m	melting temperature
TSV	Tunisian sheep virus
UTR	untranslated region

1 Introduction

1.1 Pestiviruses

The genus *Pestivirus* of the family *Flaviviridae* comprises four recognised species: *Bovine viral diarrhoea virus 1* (BVDV-1), *Bovine viral diarrhoea virus 2* (BVDV-2), *Border disease virus* (BDV), and *Classical swine fever virus* (CSFV). A fifth tentative species is *Pestivirus* of giraffe (<http://www.virustaxonomyonline.com>). The natural host of BVDV and BDV is mainly cattle and sheep/goats, respectively. Furthermore, it has been shown that BVDV can infect additional domestic animals, such as pigs (Wang *et al.*, 1996), and even wild species (Vilcek & Nettleton, 2006). BDV is found to cause natural infection of not only sheep and goats, but also cattle (Cranwell *et al.*, 2007) and swine (Vilcek & Belák, 1996). The only virus species of the *Pestivirus* genus that has not been observed to cross the species-barrier is CSFV, which is still restricted to domestic pigs and wild boars (Liess & Moennig, 1990; Moennig, 2000).

1.2 Viral infections, diseases and their importance

Pestivirus infections can vary from subclinical to manifestation of clinical signs such as: fever, diarrhoea, hemorrhagic syndrome, death, abortion (reviewed by Thiel *et al.*, 1996). Hemorrhagic syndromes with significant mortality are often seen in pigs infected with CSFV. Border disease may result in unapparent or mild clinical signs, including transient depression, pyrexia and leucopenia (Vantsis *et al.*, 1979). Lambs surviving diaplacental infection show a wide range of abnormalities, of which the hairy shaker or fuzzy-lamb syndrome is the most prominent (Rümenapf & Thiel, 2008). BVDV infection is characterized by pyrexia, anorexia,

lethargy, and profuse diarrhoea containing fresh or clotted blood, mucopurulent nasal discharge, and dehydration. Diaplacental infection may lead to abortion, stillbirth, and persistent infection of calves.

Pestiviruses belong to the main pathogens causing heavy economic losses in domestic food animals. Classical swine fever (CSF) and bovine viral diarrhoea (BVD) are notifiable to the World Organisation for Animal Health (Office International des Epizooties; OIE, see www.oie.int). CSF is a highly contagious and devastating viral disease. For example, the 1997-1998 epidemic of CSF in The Netherlands resulted in the first slaughter of over 700 000 pigs in the 429 CSF-confirmed farms. Further 1.1 million pigs at close proximity of confirmed outbreaks were preventively culled because of the risk of having been infected. For reasons of animal welfare 6.5 million weaners and adult pigs had to be killed and destroyed, whereas another 2.6 million 3-17-day-old piglets were also euthanized. In total, over 10 million pigs were destroyed at a cost of over 2 billion US dollars (reviewed by Terpstra & de Smit, 2000). Avirulent type of the disease is also observed, and has been reviewed recently (Moennig *et al.*, 2003; Rüménapf & Thiel, 2008).

Although not as catastrophic as the virulent form of CSF, BVD is considered as one of the major diseases of cattle with a worldwide economic impact. For example, the cost of BVDV infection to an average-sized dairy herd in New Zealand has been estimated at NZ\$11 334 (around € 5295) per annum or NZ\$35.19 (around € 16.4) per cow (Reichel *et al.*, 2008). Furthermore, diaplacental infection can lead to birth of persistently infected (PI) animals that serve as a reservoir for further spreading of the virus. Therefore, a key procedure of BVD eradication in Europe is focusing on identification and removal of PI animals (reviewed by Moennig *et al.*, 2005).

In addition, BVDV is an important risk factor, as it can be found as a contaminant in biological products derived from bovine serum, e.g. foetal calf serum (FCS). Falcone *et al.* (1999) reported contamination of a live infectious bovine rhinotracheitis (IBR) vaccine with a strain of BVDV-2, and the contaminating virus has been demonstrated to cause clinical signs of the disease in experimentally infected calves (Falcone *et al.*, 2003). Besides the recognised pestiviruses, “novel” bovine pestiviruses have been detected in batches of commercial FCS (Schirrmeyer *et al.*, 2004; Thabti *et al.*, 2005). Pestivirus contamination is also an issue of concern in

diagnostic and research laboratories where FCS is used as a growth supplement for cell cultures for use in virus isolation or for biological studies.

1.3 Genome organisation

The pestivirus genome is a single-stranded, positive-sense RNA molecule, without a 5' cap structure and a 3' poly (A) tail (Brock *et al.*, 1992; Moormann & Hulst, 1988). It contains two untranslated regions (UTRs) at the 5' and 3' ends, and one open reading frame (ORF) encoding a polyprotein. The polyprotein is co- and post-translationally processed into 12 polypeptides in the following order: N-terminal autoprotease (N^{pro}), capsid protein (C), envelope proteins (E^{ns}, E1, and E2), p7, and non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (reviewed by Thiel *et al.*, 1996). The 5'UTR in the BVDV genome contains two stem-loop structures that are important for efficient RNA replication (Frolov *et al.*, 1998; Yu *et al.*, 2000). The cap-independent translation initiation of viral RNAs is mediated by an internal ribosomal entry site (IRES) within the 5'UTR of the viral genome (Rijnbrand *et al.*, 1997). The 3'UTR also harbours primary and secondary RNA structures that probably function *in cis* to direct minus-strand initiation (Isken *et al.*, 2004; Pankraz *et al.*, 2005; Yu *et al.*, 1999).

As the first NS autoprotease, the N^{pro} cleaves itself from the nascent polyprotein between two conserved sites Cys-168 and Ser-169 (Stark *et al.*, 1993; Wiskerchen *et al.*, 1991). Three residues Glu-22, His-49, and Cys-69 have been shown essential for protease activity by site-directed mutagenesis studies (Rümenapf *et al.*, 1998). The N^{pro} is dispensable for virus replication except for its N-terminal part which forms part of the IRES.

The pestivirus genome encodes four structural proteins, C, E^{ns}, E1 and E2. The plus-charged C protein binds to the viral genome to form the nucleocapsid of the virion of BVDV (Murray *et al.*, 2008). However, a recent report suggests that with a compensation of mutations in the NS3, the C protein is not required for assembly of CSFV (Rümenapf *et al.*, 2008). Therefore, its true functions remain to be elucidated. The E^{ns} is a highly glycosylated protein with ribonuclease activity, homologous to the ribonuclease T2 family (Schneider *et al.*, 1993). The surface structural protein E2 forms a complex with the E1 protein and is the major viral

antigen involved in adaptive immune response (König *et al.*, 1995). The p7 is a small protein that can form ion channels, and may function in transport, maturation and release of infectious particles (Reimann *et al.*, 2008).

Six NS proteins are encoded in the 3' two-thirds of viral genome. The cysteine protease NS2 is responsible for processing of NS2-3 (Lackner *et al.*, 2004, 2006). As a multifunctional enzyme, NS3 contains a serine protease domain at the N-terminal (Bazan & Fletterick, 1989; Gorbalenya *et al.*, 1989a; Wiskerchen & Collett, 1991) and nucleoside triphosphatase and helicase domains at the C-terminus (Gorbalenya *et al.*, 1989b). Together with NS4A as its co-factor, NS3 is responsible for polyprotein processing downstream the NS3 (Xu *et al.*, 1997). NS4B may play a role in pathogenicity of BVDV (Qu *et al.*, 2001). NS5A is a phosphoprotein that associates with cellular membranes, and has been shown to interact with the α subunit of elongation factor 1 (Johnson *et al.*, 2001). NS5B is the RNA-dependent RNA polymerase (Zhong *et al.*, 1998).

1.4 Phylogeny, classification, and evolution

Pestiviruses are originally classified into three species based on the animal hosts: BDV of sheep/goats, BVDV of cattle and CSFV of pigs. As mentioned above, both BVDV and BDV are not restricted to a single host. Therefore, a pestivirus from pigs may belong to all three species. A similar situation is encountered when dividing pestiviruses based on their monoclonal antibody binding pattern, due to antigenic cross reactivity between species, and due to antigenic variation within species. Phylogenetic analysis of viral sequences has helped classification of pestiviruses. BVDV is segregated into two genotypes based on phylogenetic analysis of sequences from the 5' UTR of the viral genome (Pellerin *et al.*, 1994; Ridpath *et al.*, 1994). The “classical” BVDV strains are designated as genotype 1, whereas those detected originally from outbreaks in Northern America, associated with severe hemorrhagic syndrome, are designated as genotype 2. Both genotypes are now recognised as two species of bovine-origin: BVDV-1 and BVDV-2. Phylogenetic analyses of both partial and complete genomic sequences suggest the existence of a fifth species, termed as Pestivirus of giraffe (Becher *et al.*, 1999; Avalos-Ramirez *et al.*, 2001).

Besides the recognised species and tentative species, there are a number of pestiviruses, which have been reported but remained unclassified. Firstly, the “atypical” bovine pestivirus D32/00_‘HoBi’ was isolated from a batch of FCS originating from Brazil (Schirrneier *et al.*, 2004). Additional four pestiviruses of bovine-origin have been described: Brz buf 9, originally isolated from a buffalo in Brazil (Stalder *et al.*, 2005); CH-KaHo/cont, a cell culture contaminant possibly originating from a batch of FCS produced in South America (Stalder *et al.*, 2005); Th/04_KhonKaen, detected from serum of a calf infected naturally in Thailand (Ståhl *et al.*, 2007); and, SVA/cont-08, detected from a batch of contaminated FCS originating from South America (Liu *et al.*, submitted). All these atypical pestiviruses are closely related to each other. Secondly, two very divergent, non-bovine pestiviruses have also been reported: a pestivirus isolated from a diseased young blind pronghorn antelope in USA (Vilcek *et al.*, 2005) and Bungowannah virus from pigs (Kirkland *et al.*, 2007). Interestingly, these two viruses clustered in a well-supported clade in the phylogenetic trees based on the 5'UTR, and the N^{pro} and E2 protein sequences (Kirkland *et al.*, 2007). Thirdly, the so-called “Tunisian isolates” have been isolated from Tunisian sheep and from different batches of a contaminated Tunisian sheep pox vaccine (Thabti *et al.*, 2005). In addition, four more “Tunisian isolates”-like viruses have also been reported: 91-F-6731 and 91-F-6732 in France (Dubois *et al.*, 2008), and Aydin/04-TR and Burdur/05-TR in Turkey (Oguzoglu *et al.*, 2008). Although these viruses are typed as BDV, their closer phylogenetic relationship with CSFV rather than with BDV may merit a different classification.

Despite of a high number of studies, there is uncertainty in the classification of pestiviruses. For example, the atypical bovine pestivirus D32/00_‘HoBi’ was proposed to be a “sixth” pestivirus species according to Schirrneier *et al.* (2004). Becher *et al.* (2003) suggested that BVDV-1 and BVDV-2 are two major genotypes within a single species of BVDV. Classification of BDV is unclear: genotype-4 has been assigned to two groups of viruses of different host origin: Chamois-1 (Arnal *et al.*, 2004) and Tunisian isolates (Thabti *et al.*, 2005).

The evolutionary relationships between recognised species and unclassified pestiviruses, and among unclassified pestiviruses have not been unambiguously determined. This is particularly pronounced for relationships among atypical bovine pestiviruses, Pestivirus of giraffe, BVDV-1 and BVDV-2, as different relationships have been inferred from

analyses of different gene regions. For example, the atypical bovine pestivirus D32/00_‘HoBi’ has three different phylogenetic positions in different analyses (Schirrneier *et al.*, 2004). Furthermore, a total of nine different tree topologies have been revealed in 12 phylogenetic analyses of 52 pestiviruses using three methods: Neighbour-joining, Maximum likelihood and Bayesian approach (Liu *et al.*, unpublished). Such discrepancies in pestivirus phylogenies would simply lead to draw different or even contradictory conclusions on relationships among pestiviruses.

There is the need to clarify the uncertainties in pestivirus classification, and to outline their evolutionary relationships, which is part of the subject of this thesis.

1.5 Molecular detection of pestiviruses

Considering the pathogenic importance of pestiviruses, the economic impact of the diseases and the risks of using contaminated biological products, there is a need to develop highly sensitive methods for virus detection and identification. To meet this requirement, a number of real-time RT-PCR assays have been developed for rapid, specific and sensitive detection of pestiviruses in clinical samples as well as in biological products. For example, CSFV can be detected by real-time RT-PCR assays using a single CSFV-specific hydrolysis (TaqMan) probe (Risatti *et al.*, 2003; Hoffmann *et al.*, 2005; Ophuis *et al.*, 2006; Liu *et al.*, 2007; Zhao *et al.*, 2008; Jamnikar Ciglencecki *et al.*, 2008), and two hybridisation probes (van Rijn *et al.*, 2004). Real-time RT-PCR detection of BVDV-1 and BVDV-2 has also reported (Letellier & Kerkhofs, 2003; Young *et al.*, 2006; Baxi *et al.*, 2006). In addition, a BDV-specific real-time RT-PCR has been described by Willoughby *et al.* (2006). All these assays are useful for specific detection of the given pestivirus species in clinical specimens and to support the disease control programmes.

The high specificity is a general advantage of the real-time PCR assays. However, as a paradox, it can also be a potential weakness of the test, considering that only the target viruses are detected, while related ones may be missed due to sequence mismatches. As real-time RT-PCR assays are based on specific binding of a probe (s) to its target region in the viral genome, mismatches between the target region and primers and/or probe will adversely affect the performance of an assay. Therefore, some viruses may remain undetected by such assays. For example, the well-known ‘pan-

pestivirus' primer pair 324/326 failed to detect the atypical pestivirus D32/00_ 'HoBi' (Schirrmeier *et al.*, 2004). Uncertainties may arise from high Ct values (over 35) due to mismatches between probe and target, or due to low copies of the target.

Considering this scenario, new assays are required for the specific detection of the emerging pestiviruses (i.e., atypical bovine pestiviruses), and for the improved detection of CSFV by using new technologies in order to deal with mismatches between the probe and target region.

2 Aims of the study

The overall objectives of the present studies were to reconstruct a well-supported, reliable phylogeny for classification of recently described, unclassified pestiviruses, and for revealing the evolutionary history of pestiviruses. A further task was to develop powerful new diagnostic assays for the sensitive and specific detection of two pestiviruses, namely CSFV and atypical pestiviruses.

The specific aims were

- Application of a new strategy based on analysis of a combined sequence dataset and a new method, Bayesian approach in inferring BVDV-1 phylogeny (I);
- Recovery of a viable virus Th/04_KhonKaen from an inactivated sample and phylogenetic analysis of the full-length nucleotide sequence (II);
- Studies on phylogeny, classification and evolution of pestiviruses (III);
- Development of real-time RT-PCR assays for selective detection of atypical bovine pestiviruses using TaqMan technology, and for the improved detection of classical swine fever virus using primer-probe energy transfer system (IV & V).

3 Methodological considerations

Phylogenetic analysis of pestiviruses has been widely performed for classifying novel viruses and for revealing their evolutionary relationships. The fact that different relationships are inferred from analyses of different genetic regions points out the importance of good choice of strategy and analysis method, elements which unfortunately, are often overlooked in most studies.

3.1 Strategy

Basically, two strategies in pestivirus phylogeny could be categorised. One is based on analysis of individual genetic regions, and the other relies on the analysis of a combined sequence dataset. It is now commonly recognised that to confidently infer the evolutionary relationships of lineages, one needs to use all available data, so-called total evidence approach (Kluge, 1989). The total evidence approach acknowledges that single datasets may have systematic biases due to homoplasy, and that analysing different datasets together can bring out the hidden phylogenetic signal in them (Gatesy *et al.*, 1999; Wahlberg *et al.*, 2005). Such synergistic effects can lead to increased support for relationships, which are otherwise unsupported or weakly supported in separate gene analyses.

Before combining sequence data, incongruence-length-difference (Farris *et al.*, 1994) test should be performed to make sure that the data are not significantly heterogeneous, as combining such data can be actually detrimental to the phylogeny and can result in inaccurate relationships. The best-fit evolutionary model should also be selected and applied in the analysis.

3.2 Neighbour-joining, Maximum likelihood and Bayesian approach

Neighbour-joining is one of the distance-based methods. It is extremely fast and has been advocated for analysis of large datasets (Tamura *et al.*, 2004). However, recovery of the “true tree” is guaranteed only if the distance matrix is correct, and calculation of genetic distances is complicated by biological processes such as rate heterogeneity (Felsenstein, 2004). It is thus not recommended for use in finding a final tree (McCormack & Clewley, 2002).

Maximum likelihood (ML), one of the character-based methods, has also been used for phylogenetic analysis of pestiviruses. Under an evolutionary model, the most probable tree is found by an optimality criterion based on the character (nucleotide) at each position of a set of sequences. Disadvantages using ML are that it is computationally intensive when dealing with many taxa, and may yield unreliable results with regard to complex parameter-rich model (Holder & Lewis, 2003). The robustness of the so-called “best tree” can be estimated statistically by bootstrapping (e.g. 1000 replicates) the original dataset and a value of more than 70% is thought to indicate support for a group on the tree.

The Bayesian approach has been recently developed for inferring phylogeny (Yang & Rannala, 1997; Huelsenbeck *et al.*, 2001). It is rapidly accepted in phylogenetic studies (reviewed by Alfaro & Holder, 2006). In contrast to the traditional ML method that only gives the topology of a tree, the Bayesian analysis produces both a tree estimate and a measurement of uncertainty for the groups on the tree, thus providing a measure of support faster than ML bootstrapping. By using a Markov chain Monte Carlo (MCMC) algorithm, the Bayesian phylogenetic inference allows implementation of complex parameter-rich evolution models.

3.3 Dating of the most recent common ancestors

Dating of the most recent common ancestor (MRCA) of major lineages of pestiviruses could be explored by using the software BEAST (Drummond & Rambaut, 2007). It is a cross-platform program for Bayesian MCMC analysis of molecular sequences. The molecular clock is calibrated by the date (year of isolation) when a given sample was collected. Under the uncorrelated relaxed clock, the substitution rate is not fixed and is allowed

to vary for each lineage. When the best-fit model is applied, the programme will estimate the dates of divergence for specific MRCA.

3.4 Real-time RT-PCR

Real-time RT-PCR has been widely applied for virus detection using different chemistries/probes, such as a dual-labelled hydrolysis probe in a TaqMan assay, or a 3´end-labelled hybridisation probe in a primer-probe energy transfer (PriProET) system, and even without a probe in a CYBR Green assay. Compared to the “classical” single or nested PCR methods, the diagnostic application of the real-time PCR assays has certain advantages, such as: speed; no post-PCR handling; minimising risk of contamination; reduced hands-on time; automation of the procedure for high throughput detection and multiplexing capacity (Belák, 2007).

In a TaqMan assay, the *Taq* DNA polymerase hydrolyses the dual-labelled probe that binds to the target DNA, resulting in release of the reporter dye from the proximity of the quencher dye labelled at the 3´end of the probe. Therefore, the emitted fluorescent light from the reporter can no longer be absorbed by the quencher molecule. The accumulation of the fluorescent signal is detected and quantified by the instrument (Holland *et al.*, 1991; Heid *et al.*, 1996).

The real-time PriProET technology is initially developed as a novel tool for detection of food-and-mouth disease virus (Rasmussen *et al.*, 2003). In a PriProET assay, the 3´end-labelled probe binds to the complementary strand that the 5´ end-labelled primer binds during the annealing step. Once excited, the fluorescent light from the dye at the 5´end of the primer can excite the reporter dye at the 3´end of the probe, as both dyes are in such a close proximity that enables the Förster resonance energy transfer (FRET) to occur. The fluorescence signal is detected and quantified by the instrument.

With the emergence of new variants of pestiviruses, novel assays have to be developed for selective detection of these “new viruses”. Simultaneously, there is need for the continuous improvement of the current diagnostic methods, in order to further optimise the detection of the recognised pestiviruses.

4 Results and discussion

4.1 New strategy and method for inferring BVDV-1 phylogeny

The new strategy—analysing a combined nucleotide sequence dataset has been applied to infer BVDV-1 phylogeny, by using Bayesian approach (Paper I). BVDV-1 phylogeny was inferred from Bayesian analyses of five genetic regions of BVDV genome (5'UTR, N^{pro}, E2a, E2b, and NS3) individually and combined. Single gene analyses produced consensus trees of different topologies, where only some of the subgroups could be identified, e.g., BVDV-1a, -1b, and -1e in the tree of E2a and supported by the maximum posterior probability value (1.00). By contrast, most of the subgroups and their separation in the trees of 5'UTR, N^{pro}, E2b, and NS3 were weakly supported by posterior probability values of less than 0.95. Polytomies were found in some subgroups in four out of five consensus trees and therefore, making it difficult to resolve their relationships. In contrast to single gene analyses, analysis of the combined dataset yielded a fully resolved, well-supported consensus tree. The relationships among monophyletic subgroups 1a, 1c, 1e, and 1b could be established and supported by the maximum posterior probability value of 1.00, which were not statistically supported even in the best resolved single E2a tree. The only exception was the relationship between So CP/75 and the unnamed subgroup consisting of ZM-95, IS 25 CP/01 and IS26 NCP/01, which was weakly supported.

The overall superior performance of analysing a combined dataset over single gene analysis reflected the strength of the total evidence approach. Combining all sequence data simply increases the size of the dataset improving the phylogenetic signal. In this way, a better resolution of

phylogenetic relationships among subgroups can be achieved. Furthermore, Bayesian analysis of the combined dataset will result in only one 50%-majority-consensus tree topology with strong posterior probability support for most nodes. The relationship between subgroups could be easily interpreted based on support values.

The strategy and method being applied in this study proved to be useful for the classification of viruses into genotypes and subgenotypes, and can be used to establish reliable phylogenetic relationships among pestiviruses.

4.2 Whole-genome phylogeny of pestiviruses

A viable Th/04_KhonKaen virus was recovered from an inactivated bovine serum sample by transfection of bovine turbinate cells. The progeny virus was detected by immunoperoxidase test using BVDV-1 antiserum, which also recognizes BVDV-2 and D32/00_‘HoBi’. Cytopathic effect (CPE) was not observed on isolation and propagation of the virus, supporting the recovery of a non-cytopathogenic pestivirus from the serum sample.

The complete genome of this virus contains 12 337 nucleotides (nt), comprising 383 nt at the 5′ UTR, 254 nt at the 3′ UTR and an ORF of 11 700 nt. Neither duplication of viral sequences, nor insertions of cellular sequences were found in the genome. Comparative sequence analysis showed that this virus shares 67.3-68.3% identity with other pestivirus species, with the highest identity of 68.3% to BVDV-1 and 67.9% to BVDV-2. The genetic divergence was 42.0-43.8%, indicating a likely novel species in the *Pestivirus* genus.

Three methods, Neighbour-joining, Maximum likelihood, and the Bayesian approach were applied for phylogenetic analysis. All three methods led to a single, reliable phylogenetic hypothesis that was strongly supported by high bootstrap and posterior probability values. The recognised species BVDV-1, BVDV-2, BDV and CSFV separated into four monophyletic clades, and the tentative species *Pestivirus* of giraffe formed a single branch. Th/04_KhonKaen also formed a single branch positioned distinctly from the four established pestivirus species and from *Pestivirus* of giraffe, and closely related to BVDV-1 and BVDV-2. The position of Th/04_KhonKaen in the consensus trees was supported by bootstrap values of 99% (Neighbour-joining), 93% (ML), and posterior probability of 1.00 (Bayesian approach). This finding is important for

conclusive classification of the Th/04_KhonKaen virus within the *Pestivirus* genus as a “novel” species closely related to bovine pestiviruses.

4.3 Pestivirus phylogeny, classification and evolution

Compared with single gene analyses, whole-genome phylogeny provides conclusive genetic classification of pestiviruses. To achieve a reliable phylogeny as the basis for classification of pestiviruses, a molecular dataset of 56 pestiviruses and 2089 characters, comprising the 5'UTR and the complete N^{pro} and E2 gene regions was analysed by Maximum likelihood and Bayesian approach. Analyses of this dataset produced an identical, well-supported tree topology, regardless of the methods used. Seven monophyletic clades and two highly divergent lineages, corresponding to both recognised species and unclassified pestiviruses, could be identified. Each clade was strongly supported by the maximum posterior probability value of 1.00 and the highest bootstrap value of 100%. The relationships of the seven major clades were also supported by the maximum posterior probability value of 1.00 and by high bootstrap values of 78-99%. Therefore, this tree topology was regarded as reliable and robust.

Based on the pestivirus phylogeny, a new proposal is presented for genetic classification of pestiviruses into nine species: BVDV-1, BVDV-2, BVDV-3 (atypical bovine pestiviruses), Pestivirus of giraffe, CSFV, BDV, Tunisian sheep virus (TSV; previously termed “Tunisian isolates”), Antelope virus, and Bungowannah virus. In the new proposal, the four recognised species remain their standing positions. The tentative species, Pestivirus of giraffe may be considered as a new genotype of BVDV, based on the close evolutionary relationship with BVDV. However, efforts should be made to characterize more viruses of this group. There is no doubt that the two most divergent pestiviruses (Antelope and Bungowannah viruses) are two new species of the genus. This is supported by a comparison study that established these two pestiviruses as two new species using nucleotide sequence relatedness, serological relatedness and host of origin criteria (Ripdath *et al.*, 2008).

Schirrmeier *et al.* (2004) proposed that the atypical bovine pestivirus D32/00_HoBi should be classified as a new species, according to the above-mentioned species demarcation criteria. It is reasonable to believe that other atypical pestiviruses within the group should also be classified as

the same species based on their close phylogenetic relationship. The question, however, has been whether to coin a new species name for this taxon or to link it with an established bovine pestivirus species. There are two arguments for suggesting these atypical pestiviruses as a new genotype of BVDV. The first is the close relationship between the atypical pestiviruses and the recognised bovine pestivirus species BVDV-1 and BVDV-2, as revealed in this study through the analysis of the combined dataset. The second is the fact that the Th/04_KhonKaen virus was initially detected by a commercial BVDV Ag-ELISA Kit (Herd Check BVDV Ag/Serum plus, IDEXX Laboratories) (Kampa *et al.*, 2008), indicating a high degree of serological relatedness of the Th/04_KhonKaen virus with BVDV-1 and BVDV-2. Even in a broader context of evolution, this group of bovine pestiviruses appear to have diverged early from the common ancestor of BVDV-1 and BVDV-2, and to have evolved independently in South America and possibly South-East Asia (Thailand). Thus, in this study, the atypical bovine pestiviruses are proposed as a new species: BVDV-3.

The evolutionary relationship should also be considered as an additional criterion for species demarcation. The “Tunisian isolates”, which have been typed as a subgroup of BDV according to antigenic relatedness and host of origin criteria, are proposed in this study as representing another new species, TSV. The closer phylogenetic relationship of TSV with CSFV rather than with BDV indicated an evolutionary history of these isolates independent of BDV. Indeed, TSV shared the most recent common ancestor with CSFV rather than with BDV. Therefore, based on the evolutionary relationship of TSV with CSFV, naming these isolates either as a new genotype of BDV or as a subgroup of BDV appears inappropriate. A major concern may be the need to explain the same clinical signs of the disease caused by TSV as that of border disease. One possible explanation is that pestiviruses are closely related to each other, such that a virus of one species may cause similar or even “typical” clinical signs that are normally presented by infection with another virus species. Wang *et al.* (1996) isolated a BVDV (ZM-95 strain) from pigs with clinical signs of CSF. BVDV can also cause in sheep and goats the same signs as BDV (Rümenapf & Thiel, 2008). BDV can cause same signs in cattle as BVDV (Cranwell *et al.*, 2007). Therefore, it is not surprising to see same signs in animals infected with the proposed new species TSV as with BDV.

By dating the most recent common ancestor for the major lineages of pestiviruses, the following hypotheses concerning the “molecular clock” of pestivirus evolution were proposed. As large HPDs were associated with most of the dates, the mean MRCA date probably does not reflect the exact real date; thus, caution should be exercised when interpreting the results. The clade of mainly bovine-origin pestiviruses diverged between 1615 (HPDs, 1017 to 1904) and 1743 (HPDs, 1373 to 1926) to form the four major lineages, corresponding to the four species: Pestivirus of giraffe, BVDV-3, BVDV-2 and BVDV-1. The clade of mainly ovine- and swine-origin pestiviruses diverged between 1629 (HPDs, 1016 to 1927) and 1736 (HPDs, 1311 to 1931) to form the three species: BDV, TSV and CSFV. These pestiviruses have evolved separately and formed independent lineages after possibly being moved to specific regions of the world. For example, the first evolutionary event for pestiviruses of mainly bovine-origin was when Pestivirus of giraffe diverged and evolved independently, probably in Africa, from the common ancestor around 1615 (HPDs, 1017 to 1904). The second event was the BVDV-3 lineage that diverged in South America or Eastern Asia around 1681 (HPDs, 1210 to 1912). The third event was the separation of BVDV-2 and BVDV-1 around 1743 (HPDs, 1373 to 1926), when BVDV-2 evolved independently in North America. Finally, the type species of the *Pestivirus* genus, BVDV-1, first diverged from BVDV-2 around 1743 and then diversified around 1802, approximately 150 years before the first description of the disease in 1946 (Olafson *et al.*, 1946).

The separation of swine and ovine pestiviruses occurred at 1629 (HPDs, 1016 to 1927), when BDV speciated in ovine host and CSFV speciated in swine host, without obvious movements of the viruses. The BDV clade began diversifying around 1748 (HPDs, 1334 to 1952), approximately 210 years before the first report of the disease from the border region of England and Wales in 1959 (Hughes *et al.*, 1959). The last event of pestivirus speciation was the separation of TSV and CSFV occurring around 1736 (HPDs, 1311 to 1931). CSFV was estimated to have diversified around 1825 (HPDs, 1564 to 1947). No exact data of the first outbreak of the disease (CSF) exists, but a report of the USDA Bureau of Animal Industry from 1887–1888 indicates that the disease (then named hog cholera) was first noted in Ohio, USA, in 1833 (Liess, 1981). Other reports suggest that the disease was already present in Europe in the first part of the 19th century (Beynon, 1962). These records seem to support the

estimated age of the virus, indicating that once CSFV diversified, it soon caused disease of pigs and began being noticed.

In conclusion, the new strategy resulted in a much stable, well-supported pestivirus phylogeny, where a closer relationship between atypical bovine pestiviruses and BVDV-1 and BVDV-2 was, for the first time, unequivocally established, and statistically supported by high posterior probability and bootstrap values. The divergence times of the major pestivirus lineages were also estimated. Altogether, the analysis proved useful for a unified classification of pestiviruses.

4.4 Molecular detection of pestiviruses by real-time RT-PCR

Real-time RT-PCR assays with TaqMan technology have been widely used in veterinary medicine for detection of various pathogens (Belák, 2007). Paper IV describes a new one-step real-time RT-PCR assay for the specific detection of atypical bovine pestiviruses, including D32/00_‘HoBi’, Brzbuf 9, CH-KaHo/cont, and Th/04_KhonKaen viruses. The assay detects around 200 copies of synthetic viral RNA molecules per reaction. Coefficient variation (CV) values ranged from 0.13% to 2.11% in three tests performed within five weeks, showing that this assay is highly reproducible. To evaluate the suitability of the assay for specific detection and identification of the atypical bovine pestiviruses, it was tested on 46 clinical samples, five batches of FCS and one live *Theileria annulata* vaccine. Five clinical samples and four batches of commercial FCS tested positive for atypical pestiviruses.

By using primer-probe energy transfer technology, a novel real-time PCR assay was developed for the improved detection of CSFV. The assay is able to detect 20 copies of viral cDNA per reaction, showing a high sensitivity. The specificity has been evaluated by testing 58 pestiviruses, representing all species and unclassified pestiviruses. The assay has been found highly reproducible. One unique feature of the assay is the melting curve analysis directly following PCR amplification, which allows confirmation of the presence of the specific amplicons, and the differentiation between wild type CSFV and certain C-strain vaccines. Therefore, it could be used for the improved routine laboratory diagnosis of CSF.

As new species or novel variants of viruses are continuously emerging and posing threats to animal health and welfare, it is necessary to keep updating and modifying the diagnostic assays, and developing new techniques for the improved detection of pestiviruses. Rapid, sensitive and specific detection of these pathogens is the key step towards a better control of the diseases.

5 Conclusions

- Bayesian approach can be used for inferring BVDV-1 phylogeny, with best performance obtained from analysis of a combined nucleotide sequence dataset;
- Full-length sequence of the atypical pestivirus Th/04_KhonKaen was determined, and the phylogenetic position of this newly detected atypical pestivirus was clearly demonstrated as a sister lineage to the recognised bovine species BVDV-1 and BVDV-2 by whole-genome phylogeny;
- A reliable pestivirus phylogeny was obtained from analysis of a combined dataset by Maximum likelihood and Bayesian approach. This could be used as a basis for genetic classification of pestiviruses. The evolutionary histories of major pestiviruses were revealed;
- Real-time PCR assays, based on primer-probe energy transfer and TaqMan principles, provide powerful means for the rapid detection and molecular identification of pestiviruses, including the detection of the “newly emerging” variants in the *Pestivirus* genus.

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